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Therapeutic Effects of Human Mesenchymal Stem Cells in Ex Vivo Human Lungs Injured with Live Bacteria

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ABSTRACT. Rationale: Mesenchymal stem cells secrete paracrine factors that can regulate lung permeability and decrease inflammation, making it a potentially attractive therapy for acute lung injury. However, concerns exist whether mesenchymal stem cells' immunomodulatory properties may have detrimental effects if targeted toward infectious etiologies of lung injury.

Objective: Therefore, we tested the effect of mesenchymal stem cells on lung fluid balance, acute inflammation, and bacterial clearance. **Method:** We developed an *Escherichia coli* pneumonia model in our *ex vivo* perfused human lung to test the therapeutic effects of mesenchymal stem cells on bacterial-induced acute lung injury. **Measurements and Main Results:** Clinical grade human mesenchymal stem cells restored alveolar fluid clearance to a normal level, decreased inflammation and were associated with increased bacterial killing and **reduced bacteremia**, in part through increased alveolar macrophage phagocytosis and secretion of anti-microbial factors. Keratinocyte growth factor, a soluble factor secreted by mesenchymal stem cells, duplicated most of the anti-microbial effects. In subsequent *in vitro* studies, we discovered that human monocytes expressed the keratinocyte growth factor receptor, and that keratinocyte growth factor decreased apoptosis of human monocytes through AKT phosphorylation, an effect that increased bacterial clearance. Inhibition of keratinocyte growth factor by a neutralizing antibody reduced the anti-microbial effects of mesenchymal stem cells in both the *ex vivo* perfused human lung and monocytes grown *in vitro* injured with *Escherichia coli* bacteria. **Conclusion:** In *Escherichia coli* injured human lungs, mesenchymal stem cells restored alveolar fluid clearance, reduced inflammation and exerted anti-microbial activity, in part through keratinocyte growth factor secretion.

Word Count: 250.

Key Words: Acute lung injury, bacterial pneumonia, cell based therapy, keratinocyte growth factor.

INTRODUCTION

Despite extensive research into the pathogenesis of acute lung injury and the acute respiratory distress syndrome (ALI/ARDS), mortality remains high[1-3]. Current treatment is supportive with lung protective ventilation and a fluid conservative strategy[4, 5]. Innovative therapies are needed. Recent studies have suggested that mesenchymal stem or stromal cells (MSCs) may have therapeutic applications in multiple models of lung disease[6-17]. Despite initial interest in their multi-potent properties, engraftment in the lung does not appear to play a major therapeutic role. The beneficial effect of MSCs derives from their capacity to secrete paracrine factors that modulate immune responses and alter the responses of the endothelium or epithelium and inflammatory cells to injury[17-28]. Because of the immunosuppressive properties of MSCs, one safety concern is a potential deleterious effect on host defense against bacterial infection. Bacterial pneumonia and sepsis from a non-pulmonary cause are the two most common etiologies of ALI/ARDS[2]. However, recent studies in mice have provided evidence for the beneficial effects of MSCs in the treatment of sepsis[17, 29], although the mechanism for enhanced bacterial clearance was not clearly identified.

In the first part of this study, we hypothesized that clinical grade, cryopreserved bone marrow derived human allogeneic MSCs from a Good Manufacturing Practice facility would be as effective in restoring alveolar fluid clearance (AFC) as human MSCs cultured *in vitro* in our *ex vivo* perfused human lung injured with *E.coli* endotoxin. These clinical grade human MSCs were thawed and infused as they would be in a clinical trial. Clinical grade MSCs restored AFC rate to a normal level and decreased inflammation following endotoxin-induced ALI with similar efficacy as cultured human MSCs[30].

In the second part of these studies, we hypothesized that these same clinical grade human MSCs would be effective in an infectious model of lung injury, *E.coli* pneumonia. Intra-bronchial instillation of live *E.coli* bacteria resulted in a marked decrease in AFC and an intense pro-inflammatory response. Human MSCs administration following *E.coli* pneumonia-induced lung injury fully restored AFC, decreased inflammation and reduced total bacterial load in the injured alveolus. During the period of this study, Wu et al. reported in a mice pneumonia model that keratinocyte growth factor (KGF) stimulated the secretion of GM-CSF by alveolar epithelial cells, which activated alveolar macrophages by STAT5 phosphorylation and increased the phagocytosis and killing of Gram-negative bacteria[31]. Previously, we reported that MSCs secreted KGF and was an important paracrine mechanism through which MSCs restored vectorial ion and fluid transport in injured human alveolar epithelial type II cells[30]. To determine if KGF secretion by MSCs was involved in bacterial killing, we conducted studies with MSCs or KGF in the *ex vivo* perfused human lung and isolated blood monocytes exposed to *E.coli* bacteria.

MATERIALS AND METHODS

***Ex Vivo* Perfused Human Lung and *In Vitro* Monocyte Studies.** A detailed description of the *ex vivo* perfused human lung as previously published[30] and blood monocytes grown *in vitro* injured with *E.coli* bacteria as well as the measurement of various parameters such as AFC, percent phagocytosis of alveolar macrophages and total neutrophil and bacterial CFU counts in the alveolar fluid or conditioned medium can be found in the **Online Data Supplement**. All other techniques and reagents used are standard.

Data Analysis Plan. Results were expressed as the mean \pm SD if the data was normally distributed. Comparisons between two groups were made using the unpaired t-test. Comparisons with a sample over time were made by repeated measures of analysis of variance (ANOVA) using the Bonferroni correction for multiple-comparison testing using Statview (SAS Institute Inc.).

RESULTS

Clinical Grade Human Mesenchymal Stem Cells Restored Alveolar Fluid Clearance

Following *E.coli* Endotoxin Induced Acute Lung Injury. Similar to our prior work with cultured human MSCs[30], instillation of 5×10^6 clinical grade human MSCs processed as if in a clinical trial and given intra-bronchially (IB) or into the perfusate (IV) 1 h following endotoxin-induced ALI restored AFC to a normal level at 4 h (**Supplemental Figure E1A in the Online Data Supplement**). Instillation of normal human lung fibroblasts (NHLF) as cellular controls had no effect. Instillation of human MSCs also significantly decreased IL-1 β and IL-8 levels in the injured alveolus, indicating an anti-inflammatory effect (**Supplemental Figure E1B in the Online Data Supplement**). In addition, PKH26 labeled human MSCs preferentially migrated to endotoxin-injured lung tissue; PKH26 labeled MSCs was observed in the alveolar space 2.5 times more often in the endotoxin-injured alveoli than in the uninjured controls (**Supplemental Figure E2 in the Online Data Supplement**). To test the effect of these clinical grade human MSCs on an infectious cause of ALI, we developed an *E.coli* pneumonia model in the *ex vivo* perfused human lung (**Figure 1**).

Effect of Human Mesenchymal Stem Cells on Alveolar Fluid Clearance and Inflammatory Cell Infiltration into the Injured Lung Following *E.coli* Pneumonia. Instillation of 10^9 colony forming units (CFU) of *E.coli* markedly impaired AFC by 80%, increased the influx of neutrophils into the injured alveolus, and was associated histologically with inflammatory cells influx, thickening of the interstitium and hemorrhage at 6 h. Instillation of 5×10^6 human MSCs IB or IV 1 h following *E.coli* induced lung injury restored AFC rate to a normal level (**Figure 2A**), reduced the absolute neutrophil count in the alveolus to baseline (**Figure 2B**), and restored the lung morphology to nearly normal by histology (**Figure 2C**). Instillation of NHLF as cellular controls had no beneficial effect. Although not statistically significant by ANOVA, administration of human MSCs IV 1 h following *E.coli* pneumonia reduced the levels of inflammatory cytokines in a dose dependent manner; doubling the dose of MSCs to 10×10^6 cells reduced IL-1 β level by 63% and TNF α by 87% in the alveolar fluid as compared to *E.coli* injured lung lobes (IL-1 β : 1193 ± 549 for *E.coli*, 926 ± 156 treatment with MSC (1X), 437 ± 345 treatment with MSC (2X); TNF α : 3019 ± 1767 for *E.coli*, 1604 ± 377 treatment with MSC (1X), 384 ± 189 treatment with MSC (2X), values are mean \pm SD pg/ml, N = 3-4).

Effect of Human Mesenchymal Stem Cells on Alveolar Bacterial Load Following *E.coli* Pneumonia. Instillation human MSCs IB or IV 1 h following *E.coli* pneumonia significantly reduced the bacterial load at 6 h in the injured alveolus (**Figure 3A**). Instillation of NHLF had no effect. Doubling the dose of MSCs to 10×10^6 cells IV decreased the bacterial load by an additional 40% from *E.coli* pneumonia, suggesting a dose response. To determine if MSCs secreted anti-microbial peptides/proteins into the injured alveolus, we removed cells and

bacteria from the alveolar fluid of lungs exposed to *E.coli* with and without MSCs therapy and re-exposed this alveolar conditioned medium to *E.coli in vitro*. Alveolar fluid from lungs treated with MSCs had increased anti-microbial activity compared to the lungs exposed to *E.coli* alone (**Figure 3B**). Doubling the dose of MSCs to 10×10^6 cells IV to *E.coli* injured human lungs further increased the anti-microbial activity of the alveolar fluid.

Effect of Human Mesenchymal Stem Cells on Alveolar Macrophage Phagocytosis of *E.coli* Bacteria. In addition to the anti-microbial factors secreted by MSCs[32, 33], recent evidence showed that MSCs could increase bacterial clearance by enhancing monocyte phagocytosis[29]. Therefore, we tested the effect of MSCs on alveolar macrophage phagocytosis in the human lung injured with *E.coli*. Although there was no significant difference in total alveolar macrophage counts, instillation of human MSCs IB 1 h following *E.coli* pneumonia significantly increased the phagocytosis % and index of alveolar macrophages against *E.coli* bacteria at 6 h. Although not statistically significant, instillation of human MSCs IV 1 h following *E.coli* pneumonia also increased the phagocytosis % and index of alveolar macrophages against *E.coli* bacteria by 80% and 110% respectively at 6 h. (**Figure 4**).

Effect of Human Mesenchymal Stem Cells on Human Lung Injured with *E.coli* Pneumonia for Ten Hours. To determine if the therapeutic effect of MSCs would persist in human lungs injured with *E.coli* bacteria for a longer time period, we extended the pneumonia model to 10 h, increased the bacterial load to 10^{10} CFU of *E.coli* in order to cause bacteremia and administered the MSCs IB 2 h following the induction of injury. Similar to the results at 6 h, MSC administration restored AFC, reduced the inflammatory cell infiltration into the injured alveolus

and decreased the total bacterial load at 10 h. In addition, MSCs eliminated the bacteremia present following the more severe pneumonia (**Figure 5A-D**). In separate experiments, to determine the effect of antibiotics alone, we administered 0.2 gm ampicillin into the perfusate at 1 h. The antibiotic treatment restored most of the parameters of lung injury, but there was no effect on AFC. However, the addition of IB MSCs at 2 h following IV ampicillin at 1 h significantly restored AFC and had an additive effect in reducing the total bacterial load in both the injured alveolus and the perfusate following the induction of the pneumonia.

Effect of Human KGF on *E.coli* Pneumonia. Although not statistically significant, administration of human MSCs IB or IV following *E.coli* pneumonia increased the levels of KGF by 20% and 40% respectively in the alveolar fluid compared to *E.coli* injured lung lobes (29 ± 18 for *E.coli*, 34 ± 30 treatment with MSC IB, 40 ± 17 treatment with MSC IV (1X), values are mean \pm SD pg/ml, N =3-4). Based on our prior publication which identified KGF as a **key** paracrine secreted protein by MSCs that enhanced AFC following ALI[30] and a recent study which reported an anti-microbial effect of KGF for both **Gram** positive and negative pneumonia[31], we tested the hypothesis that recombinant human KGF would replicate some of the effects of MSCs. Similar to MSCs, KGF (100 ng) IB 1 h following *E.coli* pneumonia was as effective as human MSCs in treating several parameters of ALI: the loss of alveolar fluid clearance, increase in inflammatory cell influx into the injured **alveolus**, the increase in alveolar *E.coli* bacterial load and a decrease in alveolar macrophage phagocytosis (**Figure 6A - D**).

Effect of KGF on Blood Monocytes. Wu et al. recently demonstrated that the anti-microbial effect of KGF[31]. To determine if KGF had a direct effect on macrophage/monocyte activity

against bacteria, we exposed blood monocytes to KGF and tested their efficacy in bacterial killing. The addition of KGF to human blood monocytes increased *E.coli* bacteria killing by 20% (**Figure 7A**). This was a modest effect compared to the bacterial killing in **Figure 3A** but statistically significant. By both RT-PCR and Western Blot analyses, human blood monocytes expressed both the mRNA and protein for FGFR2, the receptor for KGF (**Figure 7B**). The addition of KGF decreased LDH release following 24 h, an effect that was associated with an increase in intra-cellular AKT phosphorylation at 1 h (**Figure 7C & D**), indicating that KGF increased the survival of monocytes, one possible additional mechanism for increased bacterial clearance. Although not statistically significant, STAT5 phosphorylation was elevated by 20% with KGF treatment. **In separate experiments, we administered several doses of KGF (10 ng/ml to 1 µg/ml) to monocytes *in vitro* and found that there was a dose dependent response on both LDH release and bacteria killing (Supplemental Figure E3 in the Online Supplement).**

Effect of Human MSCs on Blood Monocytes. Similar to the effect of KGF, the simultaneous addition of human MSCs, mixed together or separated by a Transwell plate, with human blood monocytes increased *E.coli* bacterial killing by 25% and 50%, respectively (**Figure 8A**). The level of TNF α was also decreased and the level of IL-10 was increased, suggesting an anti-inflammatory effect (**Figure 8B**). To determine if MSCs reprogrammed the monocytes to an anti-inflammatory cell with an increase in phagocytosis, gene markers of M2 phenotype were measured by RT-PCR. However, MSCs did not increase the expression of human MRC1 and Arginase on the monocytes (**Supplemental Figure E4 in the Online Supplement**). Human MSCs, whether mixed together or separated by a Transwell plate, with human blood monocytes also increased GM-CSF secretion by 17x and 6x from baseline (**Figure 8C**); **in the perfused**

human lung at 6 h, MSCs administration IB or IV at 1 h increased GM-CSF levels by 200% and 80% respectively in the injured alveolus. The addition of KGF or human MSCs also had a similar effect on human blood monocytes, cultured for 7 days to acquire the phenotype of alveolar macrophages, in terms of bacterial killing (**Figure 8D**). Finally, inhibition of KGF by a neutralizing antibody abrogated the anti-microbial effect of human MSCs in both the *ex vivo* perfused human lung and cultured blood monocytes *in vitro*, demonstrating the importance of KGF secretion in bacterial killing. In separate experiments, inhibition of GM-CSF by a neutralizing antibody also abrogated the anti-microbial effect of MSCs *in vitro*, indicating an additional paracrine factor with anti-microbial properties (**Figure 9A - C**).

DISCUSSION

The primary findings of these studies can be summarized as follows: (1) Clinical grade, cryopreserved allogeneic human MSCs were as effective as cultured MSCs in restoring AFC following *E.coli* endotoxin-induced ALI whether these MSCs were given IB or IV (**Supplemental Figure E1 in the Online Data Supplement**); (2) clinical grade human MSCs were also effective in restoring normal AFC following *E.coli* pneumonia-induced ALI whether given IB or IV (**Figure 2A**); (3) the restoration of AFC by human MSCs following *E.coli* pneumonia was associated with increased bacterial killing, explained in part by an increase in alveolar macrophage phagocytosis and secretion of anti-microbial soluble factors (**Figure 3 & 4**); (4) the therapeutic effects of human MSCs on *E.coli* pneumonia was duplicated in separate experiments with the intra-bronchial instillation of KGF, a paracrine factor secreted by MSCs and previously found to be

important for net fluid transport[30] (**Figure 6**); (5) both human MSCs and KGF in co-culture experiments increased the anti-microbial effect of human blood monocytes, which expressed the KGF receptor by RT-PCR and Western Blot analyses. To our knowledge, this is the first demonstration that KGF activates monocytes directly, not through epithelial cells, to increase the anti-microbial activity of monocytes (**Figure 7 & 8**); (6) human MSCs or KGF had a similar anti-microbial effect on monocytes grown *in vitro* for 1 week to acquire the phenotype of macrophages (**Figure 8D**); (7) inhibition of KGF by a neutralizing antibody abrogated the anti-microbial effect of MSCs on alveolar macrophages or blood monocytes against *E.coli* (**Figure 9**); (8) KGF increased AKT phosphorylation and decreased LDH release by blood monocytes **in a dose dependent manner**, suggesting an anti-apoptotic effect as an additional explanation for the increase in bacterial clearance (**Figure 7C & D & Supplemental Figure E3 in the Online Supplement**); (9) in co-culture experiments, human MSCs secreted higher levels of GM-CSF, suggesting another paracrine mechanism for the increase in bacterial phagocytosis of monocytes (**Figure 8C**); (10) MSCs *in vitro* decreased TNF α and increased IL-10 secretion by monocytes injured by LPS, indicating an immunomodulatory effect (**Figure 8B**); and **(11) in experiments in which the duration of *E.coli* pneumonia was increased to 10 h, MSC had an additive effect on injured lungs that were treated with IV antibiotics in restoring AFC and further reducing the bacterial load in the injured alveolus and perfusate (Figure 5).**

Recent studies have provided evidence for the beneficial effects of MSCs in the treatment of bacterial-induced sepsis and pneumonia. In the mouse model of sepsis, Nemeth et al. found that syngeneic MSCs reduced mortality, improved organ function and decreased total bacterial counts in the blood and peritoneal fluid, in part by the secretion of PGE₂[17]. Gonzales-Rey et al. reported the protective effect of adipose tissue-derived human and mouse MSCs in mouse

experimental colitis and sepsis, which was associated with improved bacterial clearance[34]. However, the actual mechanisms underlying enhanced bacterial clearance were not clearly identified.

Macrophages and monocytes play an important role in the production of inflammatory mediators during sepsis, and they appear to be a major cell target in the protective effect of MSCs. Mei et al. reported that the improvement in bacterial clearance in syngeneic MSCs treated septic mice could be in part explained by enhanced phagocytic activity of splenic monocytes[29]; these authors demonstrated enhancement of phagocytosis in the CD11+ cell population isolated from mouse spleen after MSCs treatment. Kim and Hematti[35] reported that human MSCs improved phagocytic activity of monocyte-derived macrophages when co-cultured *in vitro*. They reported that co-culture of human MSCs and macrophages induced an alternative state (M2) of macrophage activation, which is characterized by anti-inflammatory properties and more potent phagocytic activity. In a mouse model of peritoneal sepsis induced by Gram negative bacteria, we recently found that blood monocytes isolated from mice treated with MSC showed increased phagocytic activity compared to monocytes from control groups[36]. We also demonstrated that MSCs can inhibit bacterial growth directly in part through the secretion of antimicrobial peptide/proteins, such as LL-37 and Lipocalin-2[32, 33]. More recently, several investigators found that MSCs have Toll-like and formyl peptide-like receptors and become activated in response to different bacterial products, suggesting that MSCs may be directly involved in innate immune response[13, 37].

In this study, we identified several mechanisms underlying the anti-microbial effect of MSCs, primarily dependent on the secretion of KGF. KGF is a potent epithelial specific mitogen and differentiation factor that plays a central role in development and repair of injured epithelial

tissues[38]. KGF is produced exclusively by mesenchymal cells and act on epithelial cells through the alternatively spliced FGF-2 tyrosine kinase receptor, FGFR2-IIIb. Recombinant KGF pretreatment reduced mortality following intra-tracheal instillation of hydrochloric acid[39, 40], bleomycin[41, 42], hyperoxia[43, 44] and *Pseudomonas aeruginosa*[45]. The protective effects of KGF have been linked to stimulation of type II cell proliferation and differentiation, DNA repair, up-regulated alveolar epithelial vectorial ion and fluid transport, and enhanced surfactant lipid and protein production. More recently, Wu et al. also found that KGF enhanced the clearance of *E.coli* and *Pseudomonas aeruginosa* ALI in mice in part through the augmentation of recruitment, phagocytic activity and oxidant responses of alveolar macrophages[31]. However, in these studies, KGF treatment was only effective if given prior to or simultaneous with the injury.

In our studies, MSCs or KGF given as therapy decreased the bacterial load in the *ex vivo* perfused human lung injured by *E.coli* pneumonia (**Figure 3A & 6C**). In addition, both MSCs and KGF increased the phagocytosis and killing of both human alveolar macrophages and monocytes against *E.coli* bacteria, in part through a direct anti-apoptotic effect (**Figure 7 & 8**), and possibly through the release of GM-CSF or other anti-microbial peptide/proteins by alveolar epithelial cells or by MSCs itself (**Figure 8C**). **These beneficial effects of MSCs supplemented the benefit of antibiotics in perfused human lung injured to 10 h (Figure 5).** The importance of KGF secretion was confirmed in these experiments because administration of an anti-human KGF antibody abolished the anti-microbial effect of MSCs (**Figure 9**). Although MSCs decreased TNF α and increased IL-10 secretion by monocytes following LPS stimulation *in vitro*, suggesting an immunomodulatory phenotype (**Figure 8B**), we did not find an increase in MRC1 or arginase expression in monocytes, markers of the M2 phenotype (**Supplemental Figure E4 in the Online Data Supplement**).

There are some limitations to the current studies. It is unclear if the anti-microbial properties of MSCs are dependent on cell-cell contact or the inflammatory milieu, or both. For instance, MSCs had a more significant effect on suppressing the release of $\text{TNF}\alpha$ and increasing GM-CSF secretion by 300% *in vitro* if the cells were mixed together with the monocytes following LPS stimulation compared to separation by a Transwell plate (**Figure 8B & C**). In addition, Islam et al. recently reported that MSC formed connexin 43 gap junctional channels with LPS injured alveolar epithelium in mice, releasing mitochondria-containing microvesicles that the epithelium engulfed, which restored alveolar ATP levels and reduced lung injury[46]. The contribution of cell-mediated effects on both the alveolar epithelium and macrophages to understand the net therapeutic activity of MSC will need to be further studied to determine if MSCs are more potent than the individual secreted peptides/proteins, such as KGF.

In conclusion, clinical grade, cryopreserved allogeneic human MSCs are therapeutic in *E.coli* pneumonia in the *ex vivo* perfused human lung. The effects are mediated by the capacity of MSCs to suppress anti-inflammatory responses, restore AFC to a normal level, and to enhance bacterial clearance, including the ability to prevent translocation of bacteria into the blood stream. The anti-microbial effects of MSCs could be largely duplicated by KGF, a major paracrine product of MSCs. In both *ex vivo* and *in vitro* studies, MSCs or KGF increased alveolar macrophage or blood monocyte phagocytosis and killing of *E.coli* bacteria, explained in part by a decrease in monocyte apoptosis through AKT phosphorylation. In addition, MSCs secreted GM-CSF, a cytokine previously found to have significant anti-microbial activity[31]. These anti-microbial properties of MSCs may reduce concern for clinical testing of cell-based therapy with significant immune-suppressive effects following sepsis or pneumonia induced ALI. From the perspective of evolutionary biology, it is remarkable that MSCs have evolved the capacity to

inhibit bacterial growth, reduce acute inflammation, and enhance the function of a tight epithelial barrier that has been injured by pathogenetic bacteria.

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FIGURE LEGENDS

Figure 1. Schematic Diagram of *Escherichia coli* Pneumonia in the *Ex Vivo* Perfused

Human Lung. The right or left human lung declined for transplantation by the Northern California Transplant Donor Network is selected for perfusion if the total ischemic time is < 48 h and if the selection criteria as described in the methods are met. The lung is gently rewarmed and perfused with a crystalloid solution (DME H-21 with 5% albumin) over 1 h and oxygenated with 10 cmH₂O continuous positive airway pressure (CPAP) (FiO₂ 0.95). The perfusion rate or cardiac output is set at 0.2 liter/min and the left atrial pressure at 0 mmHg to prevent hydrostatic pulmonary edema. If the AFC rate $\geq 10\%/h$ in the control RUL or LUL, then 10^9 CFU of *E.coli* bacteria (K1 strain) is instilled into the RML or LLL and 100 ml of fresh whole blood is added to the perfusate. For MSCs treatment groups, human MSCs are instilled intra-bronchially (IB) **into the RML or LLL** or into the perfusate (IV) 1 h following the initiation of the injury.

Figure 2. Effect of Clinical Grade, Cryopreserved Allogeneic Human Mesenchymal Stem Cells on Alveolar Fluid Clearance and on Inflammatory Cell Infiltration into the Injured Lung Lobe and Histology Following *Escherichia coli* Pneumonia. (A) Instillation of clinical

grade, cryopreserved human allogeneic mesenchymal stem cells IB or IV restored the decrease in AFC in the lung lobe injured by *E.coli* pneumonia at 6 h. AFC was measured by the change in protein concentration of a 5% albumin instillate in the lung lobe over 1 h and expressed as mean AFC (%/h, per 150 ml of BAL) \pm SD for each condition. **N = 20 for control lobe, N = 4 for *E.coli* injured lung lobe, N = 3 for *E.coli* injured lung lobe treated with MSCs IB or IV or NHLF, * P < 0.008 compared to controls by ANOVA (Bonferroni).** (B) Instillation of clinical grade MSCs IB or IV decreased the influx of inflammatory cells, specifically neutrophils, into the lung lobe injured

by *E.coli* pneumonia at 6 h. Absolute neutrophil counts are expressed as mean total neutrophil counts ($\times 10^7$ cells) \pm SD for each condition. **N = 20 for control lobe, N = 4 for *E.coli* injured lung lobe, N = 3 for *E.coli* injured lung lobe treated with MSCs IB or IV or NHLF**, * $P < 0.0001$ compared to controls by ANOVA (Bonferroni), $\sqrt{P} < 0.0002$ vs. *E.coli* pneumonia by ANOVA (Bonferroni). There was no statistical difference in the absolute alveolar macrophage counts between groups. **(C)** Human lungs exposed to *E.coli* bacteria with and without MSCs were fixed in 10% formalin at 6 h. Sections were stained with hematoxylin and eosin. The administration of human MSCs 1 h after *E.coli* pneumonia injury reduced the level of hemorrhage, edema and cellularity in the injured lung lobe at 6 h.

Figure 3. Effect of Allogeneic Human Mesenchymal Stem Cells on Alveolar *Escherichia coli* Bacterial Load Following *Escherichia coli* Pneumonia. **(A)** Instillation of clinical grade, cryopreserved human allogeneic mesenchymal stem cells IB or IV decreased the total bacterial load in the lung lobe injured by *E.coli* pneumonia at 6 h. Total bacterial counts were expressed as mean ($\times 10^6$ CFU counts/ml) \pm SD for each condition. **N = 18 for control lobe, N = 3 for *E.coli* injured lung lobe with or without administration of MSCs IB or IV or NHLF**, * $P < 0.0005$ vs. control CFU counts/ml by ANOVA (Bonferroni), $\sqrt{P} < 0.0001$ vs. *E.coli* pneumonia CFU counts/ml by ANOVA (Bonferroni). **(B)** The alveolar fluid conditioned medium of lung lobes treated with human MSCs IB or IV had increased anti-microbial activity against *E.coli* bacteria re-exposed *in vitro*. Total bacterial counts were expressed as mean ($\times 10^4$ CFU counts/ml) \pm SD for each condition. **N = 19 for control lobe, N = 4 for *E.coli* injured lung lobe, N = 3 for *E.coli* injured lung lobe treated with MSCs IB or IV or NHLF**, * $P < 0.0005$ vs. control CFU counts/ml by ANOVA (Bonferroni).

Figure 4. Effect of Allogeneic Human Mesenchymal Stem Cells on Alveolar Macrophage Phagocytosis of *Escherichia coli* Bacteria. (A) A representative alveolar macrophage found from cytopsin slides of bronchoalveolar lavage fluid from the injured alveolus under different treatment conditions: IB MSCs, IV MSCs and IB KGF. (B) The instillation of human MSCs IB 1 h following *E.coli* pneumonia significantly increased the percent phagocytosis and phagocytosis index of alveolar macrophages against *E.coli* bacteria at 6 h. Although not statistically significant, the instillation of human MSCs IV 1 h following *E.coli* pneumonia increased the percent phagocytosis and phagocytosis index of alveolar macrophages against *E.coli* bacteria by 80% and 110% respectively at 6 h. The percent phagocytosis (1) and phagocytosis index (2) were calculated by (1) quantifying the number of alveolar macrophages containing bacteria out of 100 macrophages from the bronchoalveolar lavage fluid of 3 different lung preparations per condition and (2) quantifying the average number of bacteria per macrophage in 100 macrophages containing the bacteria from 3 different lung preparations per condition. The values are expressed as mean \pm SD for each condition. N = 3. * P< 0.05 for % phagocytosis and * P< 0.03 for phagocytosis index.

Figure 5. Effect of Antibiotics and/or Human Mesenchymal Stem Cells on *Escherichia coli* Pneumonia at Ten Hours. We extended the *ex vivo* perfused human lung model injured with *E.coli* pneumonia to 10 h to determine if MSC treatment would be therapeutic if given at a later time-point. We also increased the *E.coli* IB dose to 10^{10} CFU from 10^9 CFU to cause a transient bacteremia in the perfusate. Similar to the previous set of experiments at 6 h, IB MSC restored all the parameters of lung injury when given 2 h following the induction of *E.coli* pneumonia: (A)

the loss of alveolar fluid clearance. AFC is expressed as mean AFC (%/h, per 150 ml of BAL) \pm SD for each condition. N = 12 for control lobe, N = 3 for *E.coli* injured lung lobe treated with or without administration of MSCs or ampicillin IV \pm MSCs, * P<0.0001 vs. control, $\sqrt{}$ P<0.0.0001 vs. *E.coli* and # P<0.0005 vs. *E.coli* + IV Amp AFC by ANOVA (Bonferroni); **(B)** the influx of neutrophils into the injured alveolus. Absolute neutrophil counts are expressed as mean total neutrophil counts ($\times 10^7$ cells) \pm SD for each condition; **(C)** the increase in the total bacterial CFU counts in the injured alveolus. Total bacterial counts were expressed as mean ($\times 10^6$ CFU counts/ml) \pm SD for each condition. * P<0.002 vs. control CFU counts/ml by ANOVA (Bonferroni); **(D)** the bacteremia present in the perfusate. The highest bacterial count found in the perfusate/h was expressed as mean ($\times 10^6$ CFU counts/ml) \pm SD for each condition. * P<0.0001 vs. control, $\sqrt{}$ P<0.004 vs. *E.coli* CFU counts/ml by ANOVA (Bonferroni). Treatment of *E.coli* pneumonia with ampicillin (0.2 gm) IV at 1 h restored most of the parameters of ALI similar to MSCs except for the restoration of AFC **(A-D)**. The addition of IB MSC at 2 h given following IV Amp at 1 h following *E.coli* pneumonia had an additive effect in restoring AFC rate in the injured alveolus as well as decreasing the total bacterial load in both the alveolus and perfusate **(C-D)**.

Figure 6. Effect of Recombinant Human Keratinocyte Growth Factor on *Escherichia coli* Pneumonia. KGF instillation (100 ng) IB 1 h following *E.coli* pneumonia restored many of the parameters of ALI similar to MSCs: **(A)** Instillation of KGF IB restored the decrease in AFC in the lung lobe injured by *E.coli* pneumonia at 6 h. AFC is expressed as mean AFC (%/h, per 150 ml of BAL) \pm SD for each condition. N = 20 for control lobe, N = 3-4 for *E.coli* injured lung lobe treated with or without KGF, * P<0.009 vs. control AFC by ANOVA (Bonferroni); **(B)** Instillation of

KGF IB decreased the influx of neutrophils into the injured lung lobe at 6 h. Absolute neutrophil counts are expressed as mean total neutrophil counts ($\times 10^7$ cells) \pm SD for each condition. **N = 20 for control lobe, N = 4 for *E.coli* injured lung lobe treated with or without KGF**, * $P < 0.0001$ vs. control by ANOVA (Bonferroni), $\sqrt{P} < 0.006$ vs. *E.coli* pneumonia by ANOVA (Bonferroni); **(C)** Instillation of KGF IB decreased the total bacterial load in the injured lung lobe at 6 h. Total bacterial counts were expressed as mean ($\times 10^6$ CFU counts/ml) \pm SD for each condition. **N = 18 for control lobe, N = 3 for *E.coli* injured lung lobe with or without KGF**, * $P < 0.007$ vs. control CFU counts/ml by ANOVA (Bonferroni), $\sqrt{P} < 0.0001$ vs. *E.coli* pneumonia CFU counts/ml by ANOVA (Bonferroni); and **(D)** Instillation of KGF IB 1 h following *E.coli* pneumonia significantly increased the percent phagocytosis and phagocytosis index of alveolar macrophages against *E.coli* bacteria at 6 h. The values are expressed as mean \pm SD for each condition. N = 3. * $P < 0.03$ for % phagocytosis and * $P < 0.05$ for phagocytosis index.

Figure 7. Effect of Recombinant Keratinocyte Growth Factor on Peripheral Blood

Monocytes. (A) The simultaneous addition of KGF (100 ng/ml) to isolated human blood monocytes increased *E.coli* bacteria killing by 20%. Total bacterial counts were expressed as mean (% of control) \pm SD for each condition. N = 14-16, * $P < 0.0001$ vs. control CFU count; **(B)** By both RT-PCR and Western Blot analyses, human blood monocytes were found to express both the mRNA and protein for FGFR2, the receptor for KGF. Primary cultures of human alveolar epithelial type II cells (TII) **were** used as a positive control; **(C & D)** The addition of KGF decreased LDH release following 24 h, which was associated with an increase in intra-cellular AKT phosphorylation at 1 h. LDH released was expressed as mean (% of total LDH) \pm SD for each condition. N = 20-24, * $P < 0.0008$ vs. control LDH release; the ratio of phosphorylated

AKT/Total AKT was expressed as mean (% of control) \pm SD for each condition. N = 10, * P<0.03 vs. control P-AKT/Tot AKT ratio. The ratio of phosphorylated STAT5/Total STAT5 was expressed as mean (% of control) \pm SD for each condition. N = 10, * P>0.05 vs. control P-STAT5/Tot STAT5 ratio.

Figure 8. Effect of Human Mesenchymal Stem Cells on Peripheral Blood Monocytes. (A)

The simultaneous addition of human MSCs, mixed together or separated by a Transwell plate, with isolated human blood monocyte increased *E.coli* bacteria killing. Total bacterial counts were expressed as mean (% of control) \pm SD for each condition. N = 8-15, * P<0.0002 vs. control CFU counts; **(B)** In addition, the level of TNF α was decreased and the level of IL-10 was increased, suggesting an anti-inflammatory effect. The TNF α level was expressed as mean (% of control) \pm SD for each condition. N = 8-11, * P<0.02 vs. control and $\sqrt{}$ P<0.0001 vs. MSCs (Transwell) for TNF α level. The IL-10 level was expressed as mean (% of control) \pm SD for each condition. N = 8-11, * P<0.03 vs. control for IL-10 level. **(C)** Human MSCs, whether mixed together or separated by a Transwell plate, with human blood monocytes increased GM-CSF secretion by 17x and 6x from baseline. The GM-CSF level was expressed as mean (% of control) \pm SD for each condition. N = 8-15, * P<0.003 vs. control and $\sqrt{}$ P<0.0001 vs. MSCs (Transwell) for GM-CSF level. **(D)** The addition of KGF or human MSCs had the same anti-microbial effect on monocytes cultured for 7 days to take on the phenotype of alveolar macrophages. Total bacterial counts were expressed as mean (% of control) \pm SD for each condition. N = 4, * P<0.05 vs. control CFU counts/ml and $\sqrt{}$ P<0.05 vs. KGF CFU counts/ml.

Figure 9. Effect of Anti-Human Keratinocyte Growth Factor or Anti-Granulocyte Macrophage Colony-Stimulating Factor Neutralizing Antibody on the Effect of Human Mesenchymal Stem Cells on Alveolar Macrophages and Peripheral Blood Monocytes. The inhibition of KGF by a neutralizing antibody abrogated the anti-microbial effect of human MSCs on (A) alveolar macrophages in the *ex vivo* perfused human lung and (B) blood monocytes cultured *in vitro*. (C) Similar to anti-KGF Ab, the inhibition of GM-CSF by a neutralizing antibody abrogated the anti-microbial effect of human MSCs *in vitro*. Total bacterial counts in the alveolar fluid for the perfused human lung were expressed as mean ($\times 10^6$ CFU counts/ml) \pm SD for each condition. N = 3-5, * P<0.05 vs. MSCs CFU counts/ml treated with Goat Control IgG. Total bacterial counts for monocytes grown *in vitro* were expressed as mean (% of control) \pm SD for each condition. N = 8-9. For anti-KGF Ab, * P<0.05 vs. MSCs treated with Goat Control IgG. N = 14-16. For anti-GM CSF Ab, * P<0.04 vs. MSCs treated with Goat Control IgG.

Supplemental Figure E1. Effect of Clinical Grade, Cryopreserved Allogeneic Human Mesenchymal Stem Cells on Alveolar Fluid Clearance Following *Escherichia coli* Endotoxin Induced Acute Lung Injury. (A) Instillation of clinical grade, cryopreserved human allogeneic mesenchymal stem cells IB or IV restored the decrease in AFC in the lung lobe injured by *E.coli* endotoxin at 4 h. AFC was measured by the change in protein concentration of a 5% albumin instillate in the lung lobe over 1 h and expressed as mean AFC (%/h, per 150 ml of BAL) \pm SD for each condition. N = 12 for control lobe, N = 6 for endotoxin, N = 3 for endotoxin injured lung lobe treated with or without administration of MSCs IB or IV or NHLF, * P<0.0001 vs. control AFC by ANOVA (Bonferroni), $\sqrt{}$ P<0.0001 vs. LPS (0.1 mg/kg) AFC by ANOVA

(Bonferroni). **(B)** Instillation of clinical grade MSCs IB or IV restored the increase in cytokines in the alveolar fluid of the lung lobe injured by *E.coli* endotoxin at 4 h. N = 14 for control lobe, N = 8 for endotoxin, N = 3 for endotoxin injured lung lobe with or without administration of MSCs IB or IV, * P<0.0001 vs. control by ANOVA (Bonferroni), $\sqrt{}$ P<0.0001 vs. LPS (0.1 mg/kg) by ANOVA (Bonferroni) for IL-1 β . * P<0.0001 vs. control by ANOVA (Bonferroni), $\sqrt{}$ P<0.0001 vs. LPS (0.1 mg/kg) by ANOVA (Bonferroni) for IL-8.

Supplemental Figure E2. Trafficking of PKH26 Labeled Mesenchymal Stem Cells in the Ex Vivo Perfused Human Lung Injured with *Escherichia coli* Endotoxin. Although not statistically significant, PKH26 labeled MSCs preferentially trafficked into the alveolar space injured with *E.coli* endotoxin than to the control uninjured lung lobe. The movement of the labeled MSCs was not effected by the level of perfusion of the lung lobe. The number of MSCs in the alveolar space in the control lung lobe was the same whether the control lobe was the RUL or the RLL. N =3.

Supplemental Figure E3. Dose Dependent Effect of Recombinant Keratinocyte Growth Factor on Peripheral Blood Monocytes. (A) The addition of increasing amount of KGF decreased LDH release in a dose dependent manner following 24 h. LDH released was expressed as mean (% of total LDH) \pm SD for each condition. N = 15-21, * P<0.001 vs. control LDH release by ANOVA (Bonferroni), $\sqrt{}$ P<0.0006 vs. LPS + 1 μ g/ml LDH release by ANOVA (Bonferroni). **(B)** The simultaneous addition of increasing amounts of KGF (10 ng/ml to 1 μ g/ml) to isolated human blood monocytes increased *E.coli* bacteria killing in a dose dependent manner. Total bacterial counts were expressed as mean (% of control) \pm SD for each condition.

N = 6 for KGF at 10 ng/ml and N = 15 for all other groups, * $P < 0.008$ vs. control CFU count by ANOVA (Bonferroni);

Supplemental Figure E4. Effect of Allogeneic Human Mesenchymal Stem Cells on Expression of Markers for M2 Monocyte Phenotype. Blood monocytes co-cultured with human MSCs did not increase the expression of mannose receptor C type 1 (MRC1) or arginase by RT-PCR. Both cellular markers are representative of the M2 or the anti-inflammatory phenotype of monocytes/macrophages. N=4.